Journal of the Science of Food and Agriculture *J Sci Food Agric* **86**:2266–2272 (2006)

Perspective

Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods

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Abstract: Functional foods are defined as foods or food components that provide health benefits beyond basic nutrition (for the intended population). Increased interest in bioactive food components and phytochemicals has arisen from numerous epidemiological studies that suggest that certain phytochemicals can reduce risk of chronic diseases. Sales of functional foods containing high concentrations of bioactive components have increased dramatically during the past two decades. This paper illustrates with examples the significance of sample preparation in developing analytical methodologies for accurate estimation of potentially bioactive compounds present in functional foods using phenolic phytochemicals as model substrates. The primary three steps in any analysis are sampling, sample preservation, and sample preparation. These three initial steps are often overlooked and considered 'as a means to an end' and are often not well documented in the published literature. This paper outlines a systematic protocol for optimizing extraction of phytochemicals from different plant matrices. Accurate analysis of bioactive compounds is critical for their precise and reproducible quantification in different foods, establishing appropriate dietary intake and safety guidelines, and understanding their role in human health and nutrition.

Keywords: functional foods; optimization of extraction technique and conditions; phenolic compounds; sample preparation

FUNCTIONAL FOODS

Functional foods are defined as foods or food components that provide health benefits beyond basic nutrition (for the intended population). Functional foods may improve health in general, reduce impact of illness, delay onset of disease, treat disease in progress, or even cure disease.[1] Thus functional foods blur the distinction between a food and a drug and serve as a bridge to link these two distinct categories. Examples include calcium-fortified orange juice for maintaining bone health, omega-3 fat-substituted foods or food products for protecting against certain forms of cancer and cardiovascular diseases and margarines formulated with cholesterol-lowering stanols.[2–4] Even oatmeal,[4] cranberry juice,[4] soybean,[5] broccoli,[6,7] green tea,[8] and flax seed[9] might be considered as functional foods as they are shown to contain dietary components that may reduce the risk of certain chronic diseases.

The idea of functional foods is not new. Hippocrates proclaimed over 2500 years ago, 'Let food be thy medicine and medicine be thy food.'[10] The term 'functional food' was first introduced in Japan in the mid 1980s[11] and it first appeared in English in an article in *Nature* (1993) with the headline 'Japan explores the boundary between food and medicine'.[12] Since then several new terms such as nutraceuticals, pharmafoods, designer foods, novel foods, and medical foods have been introduced and used as alternatives for functional foods.[13] Between two and seven million hits were detected when functional foods or related terms were searched on the Internet using a Google web browser. In the East, the concept of functional food has been an integral part of cultures for centuries: however, in the West, the idea of functional foods is relatively new and has seen an explosive growth during the past two decades. Although functional foods are not well

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(*Received 13 March 2006; revised version received 18 May 2006; accepted 31 July 2006*)
Published online 28 September 2006; DOI: 10.1002/jsfa.2666

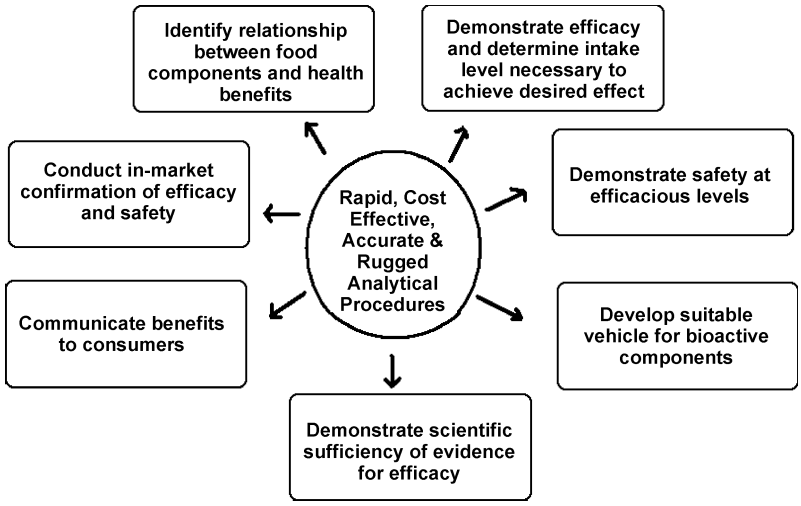

Figure 1. Institute of Food Technologist expert panel report that describes a seven-step process (see boxes) to ensure that functional foods are safe and effective, with health claims that are accurate, and regulatory oversight that is science based and efficient.

defined by the industry, global sales of approximately \$55.5 billion were reported in the year 2001.[14] In the USA, the functional foods market grew by 9.1% to sales of \$20.6 billion in 2002 and *Nutrition Business Journal* expects that by 2010 functional foods sales will be approximately \$31.2 billion.[14,15] This increase in demand for functional foods has been primarily attributed to several factors, such as increase in health care costs, increase in scientific evidence that diet can alter disease prevalence and progression, consumer interest, awareness and desire to enhance personal health, and changes in food regulations.[16]

NEED FOR ACCURATE ANALYSIS

Recently, the Institute of Food Technologists (IFT) expert panel published a report on functional foods which describes seven key steps for designing, developing, and marketing value-added functional foods (Fig. 1).[17] Development of rapid, rugged, and accurate analytical procedures, though not directly emphasized in the IFT expert panel report, is critical for the success of most of the steps described in Fig. 1. Accurate identification of bioactive compounds is essential to identify relationships between different food components and their health benefit. In addition, precise quantitation is critical to determine dietary intake levels and safety guidelines for potentially bioactive compounds necessary to achieve desired health-beneficial properties. As the influence of functional foods is often not direct, it may require a much longer time period to show a beneficial health effect as compared to a pharmaceutical drug. Novel, cost-effective, and rapid analytical methods are also needed to monitor the biomarkers influencing the disease state to effectively demonstrate benefits of these value-added foods to consumers. Further, assay procedures should be harmonized at international levels to differentiate and facilitate trade of these value-added functional foods in the global market.

PHENOLIC COMPOUNDS

Phenolic compounds are ubiquitously distributed throughout the plant kingdom. There is increasing evidence from epidemiological, *in vivo*, *in vitro*, and clinical trials clearly suggesting that the phenolic compounds present in fruits, vegetables, and grains may reduce risk of chronic diseases such as cancer, anti-inflammatory, cardiovascular, and neurodegenerative diseases.[18–21] Over 8000 phenolic compounds with diverse structural configurations and polarities have been isolated and reported from plant sources.[22] Phenolic compounds can be chemically classified into three major categories: simple phenols, polyphenols, and a miscellaneous group (Fig. 2). Simple phenols primarily consist of phenolic acids (cinnamic acid and benzoic acid derivatives). However, polyphenols are further subdivided into two main classes: tannins (polymers of phenolic acids, catechins, or epicatechins) and flavonoids (flavones, isoflavones, anthocyanins, chalcones, flavonol, flavanones, etc.). The third, miscellaneous group consists of other phenolic compounds such as coumarins, stilbenes, and lignans.[23]

FOUR STEPS OF ANALYSIS

The four common steps for any analytical method are sampling, sample preservation, sample preparation, and analysis (separation and detection).[24] Over 90% of the development made during the past few decades has focused on the final analysis step. Remarkable developments in instrumentation, spectroscopy, and chromatography have resulted in rapid advancement of methods for high-throughput separation and detection of complex multi-component mixtures containing trace quantities of the analytes of interest. However, there has been limited research in the area of sampling, sample preservation and sample preparation, which are the basic foundations for developing a quality and rugged analytical

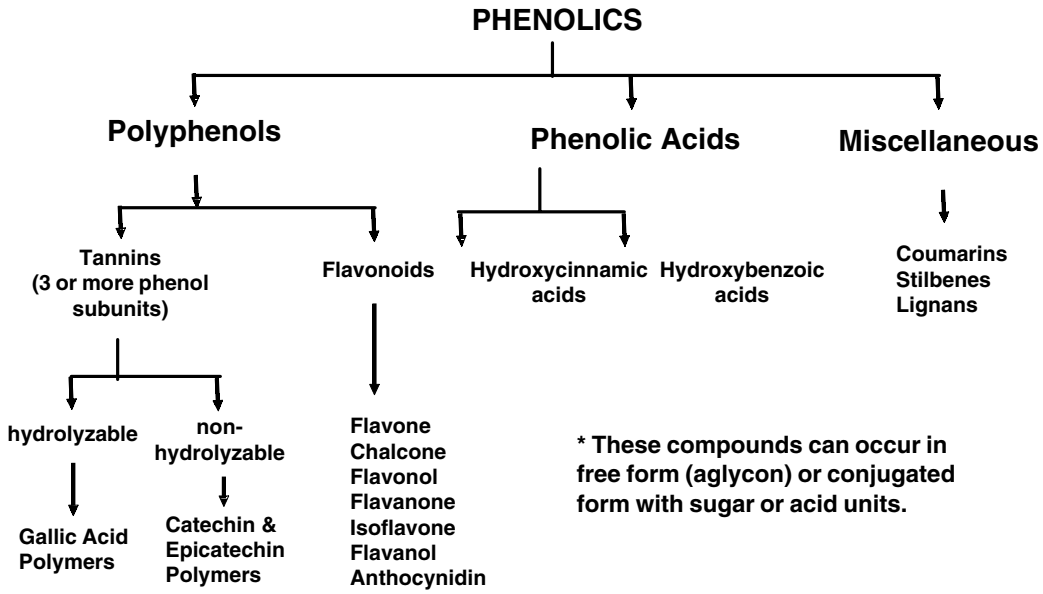

Figure 2. Classification of phenolic compounds.

procedure. It is well documented in the literature that approximately 60% of analysis time is spent in sample preparation and around 30% of analytical error stems from the sample preparation step.[25,26] This paper illustrates with examples the importance of the first three steps (sampling, sample preservation, and sample preparation) using phenolic micronutrients as model substrates.

Sampling

The initial step in any analysis is sampling, where a representative sample is collected from the entire sample matrix that needs to be analyzed. The sample is obtained in such a way that it truly represents the entire sample. It is often observed in published manuscripts that details about the samples are poorly documented. Authors often overlook and fail to provide information regarding what part of the fruit, vegetable or plant material was used during analysis; for example, whether peels or seeds of the fruits or vegetable were included as a part of sample during analysis or only the flesh or edible part was used. The significance of documenting details regarding sampling is well illustrated by the example presented in Table 1 depicting the glycosylated flavonoid content from methanol extracts of peels and seeds of two citrus fruits. The total phenolic content of citrus fruit peels was between 8 and 20 times higher than that of the same citrus fruit seeds. In addition, the phenolic profile of different parts of the same citrus fruit also varied significantly as both glycosylated flavanones, neoeriocotrin and narirutin, were detected in measurable amounts only in peels of the citrus fruit and not in seeds.[27] In a recent study, Andaur *et al*. showed that there is significant variation in the sugar content and berry size collected from the different locations (top, middle, and bottom) within the same grape cluster bunch.[28]

Sample preservation

This is an important step as there is often some delay between sample collection and analysis. Proper sample preservation ensures that the sample retains its physical and chemical characteristics from the time it is collected to the time it is analyzed.[24] Table 2 provides an excellent example depicting the influence of sample preservation on assay of the phenolic compounds from broccoli samples.[29] Over 50% reduction in the levels of three components, namely total flavonoids, total caffeoyl-quinic and total sinapic and feruloyl derivatives, were obtained when samples were either stored at 1 °C for 7 days or at 15 °C for 3 days. Therefore it is essential to inactivate all enzymatic, metabolic, and chemical reactions during the sample preservation step to maintain accurate sample identity, and demonstrate the effectiveness of such procedures

Table 1. Influence of sampling on glycosylated flavanone content (mg g^{-1} of dry matter) in methanol extract of citrus fruits

Extract	Eriocitrin	Neoeriocitrin	Narirutin	Naringin	Hesperidin	Neohesperidin	Total
Seeds							
Lemon	1.61 ± 0.19			0.04 ± 0.006	0.50 ± 0.03		2.15
Sour orange				0.77 ± 0.11		0.25 ± 0.01	1.02
Peels							
Lemon		6.12 ± 0.07		6.06 ± 0.14		4.37 ± 0.22	16.55
Sour orange	Trace	3.80 ± 0.27	0.25 ± 0.05	10.97 ± 0.38	0.66 ± 0.11	6.62 ± 0.54	22.3

J Sci Food Agric **86**:2266–2272 (2006)
DOI: 10.1002/jsfa

Table 2. Influence of sample preservation on total flavonoids, total caffeoyl-quinic derivatives and total sinapic and feruloyl derivatives in broccoli samples

Temperature	Time	Total flavonoids (mg kg^{-1} FW[a])	Caffeoyl-quinic derivatives (mg kg^{-1} FW)	Sinapic and feruloyl derivatives (mg kg^{-1} FW)
	At harvest	532.7	86.8	151.5
1 °C	7 days	205.1	23.6	74.7
% loss		61.5%	72.8%	50.7%
15 °C	3 days+	217.5	19.1	85.0
% loss		59.2%	74.4%	43.9%

[a] FW, fresh weight.

in any report. Thus researchers need to study in detail the influence of storage temperature on the analyte of interest and preserve samples under appropriate conditions.

Sample preparation

This step may consist of multiple steps such as sample drying, homogenization, sieving, extraction, pre-concentration, derivatization, and hydrolysis. The motive behind sample preparation can be multi-fold: to increase the efficiency of an assay procedure, to eliminate or reduce potential interferences, to enhance the sensitivity of the analytical procedure by increasing the concentration of the analyte in the assay mixture, and sometimes to convert the analyte of interest to a more suitable form that can be easily separated, detected, and/or quantified.

Phenolic compounds are known to exist as free aglycons or as conjugates with sugars or esters, or as a polymer with multiple monomeric units.[30–32] It is also well documented that phenolic compounds are not uniformly distributed and may be associated with other cellular components such as cell walls, carbohydrates, or proteins. In addition, the stability of phenolic compounds varies significantly; some are relatively stable and others are thermally labile, unstable, and easily prone to oxidation.[30–32] Therefore it is practically impossible to develop an efficient and uniform method for extraction of all phenolic compounds with a single solvent system, as polarities of different phenolic compounds vary significantly due to their conjugation status and their association with the sample matrix. Thus optimization of the sample preparation procedure is essential for the accurate estimation of phenolic compounds present in different food matrices.

Table 3 shows the content of some phenolic compounds for some food materials as reported in the USDA nutrient database.[33] It is clearly evident that there is a wide variation in the reported phenolic compounds obtained by different researchers. These variations can be partially attributed to the variation in genotype,[34,35] storage[29,36] and/or environmental conditions.[37,38]

Some of the differences in assay of phenolic compounds may also stem from the variation in sample preparation procedure.[39] The influence of sample preparation on assay of phenolic acid from eggplant samples was clearly illustrated in our recent publication[39] that compared various extraction procedures as applied by several researchers for extraction of phenolic compounds from eggplant samples (Fig. 3). All extraction experiments were performed with the same homogenized and freeze-dried eggplant sample (Black Bell cultivar) in our own laboratory with very similar procedures as documented in published manuscripts. As the same homogenized eggplant sample was used for all extraction experiments, the variations due to genotype, storage, and/or environmental conditions were eliminated.

Significant variations in the yields of chlorogenic acid and total phenolics were obtained when extractions were performed by the different reported procedures. All extracts were assayed by high-performance liquid chromatography (HPLC) (phenolic acids) and Folin–Ciocalteu (total phenolics) methods, respectively. Optimum yields of chlorogenic acids (HPLC)

Table 3. Flavonoid content of selected foods, showing large variations in the phenolic content as reported in the USDA database

Nutrient database #	Sample	Subclass	Flavonoid	Mean (mg 100 g^{-1}, edible portion)	# of samples	Minimum (mg 100 g^{-1}, edible portion)	Maximum (mg 100 g^{-1}, edible portion)	# of references providing data
11233	Kale (raw)	Flavonol	Kaempferol	26.74	8	0.48	47.00	4
09078	Cranberries (raw)	Flavonol	Quercetin	14.02	16	7.30	25.00	4
09206	Orange juice	Flavanone	Hesperetin	12.54	161	4.93	39.20	7
11297	Parsley (raw)	Flavone	Apigenin	302.00	5	0.00	630.00	3
99070	Tea, green (brewed)	Flavan-3-ol	Epicatechin	8.47	62	1.90	26.00	8
99071	Tea, oolong (brewed)	Flavan-3-ol	Epicatechin 3-gallate	36.01	15	7.36	71.10	3

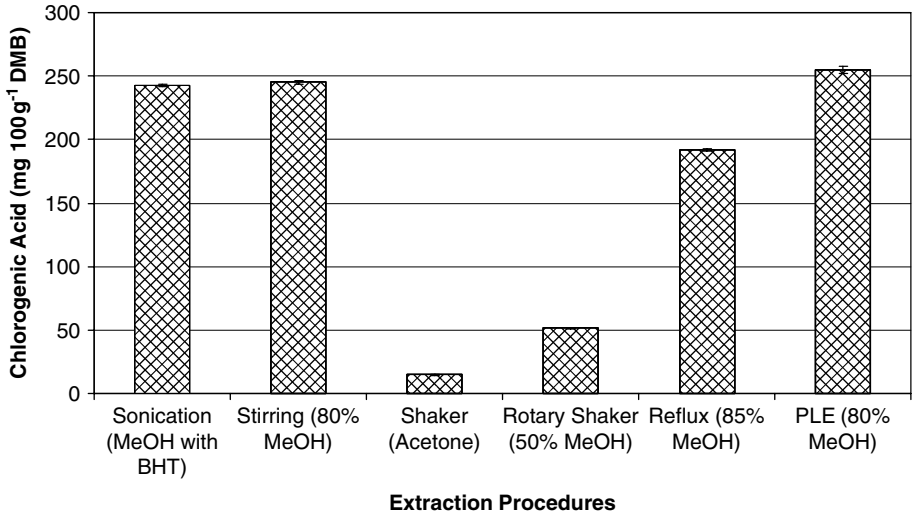

Figure 3. Comparison of extraction of chlorogenic acid (mg 100 g^{-1} dry matter basis) from an eggplant (Black Bell cultivar) sample performed in our laboratory with different reported extraction procedures/conditions as cited in the literature. All extracts were analyzed by HPLC with a diode array detection method.

and total phenolics (Folin–Ciocalteu) from the Black Bell cultivar of eggplant were obtained when extractions were performed with mixture of MeOH:H_2O at a ratio of 80:20 (% v/v) using a pressurized liquid extractor (PLE) at 100 °C. The quantity of chlorogenic acid and total phenolics extracted from the same eggplant samples by the previously reported procedures using a wrist shaker, rotary shaker, stirring, sonication, or reflux with different extraction solvents (acetone or varying composition of MeOH:H_2O solvent mixtures) ranged from 5% to 95% as compared to PLE.

The variation in the percentage of chlorogenic acid extracted using different extraction procedures from the same eggplant sample unambiguously illustrates that sample preparation directly influences extraction of phenolic compounds from different matrices. In another study, Keinanen and Julkunen-Tiitto showed that quantity of phenolic compounds extracted from birch leaves (*Betula pendula* Roth) were affected by the sample drying procedure.[40] The authors compared the influence of eight different sample drying conditions (air drying at ambient temperature, oven-drying at 40 °C, oven-drying at 80 °C, freeze-drying after pre-freezing samples with liquid N_2, freeze-drying after pre-freezing samples at −18 °C, freeze-drying without pre-freezing samples, storing frozen samples for 12 days without drying, and immediate extraction of fresh samples) on extraction of phenolic compounds from birch leaves. The authors recommended that for quantitative analysis samples should be immediately analyzed after collection. Among the drying procedures, highest concentrations of phenolics were obtained with freeze-drying samples after pre-freezing at −18 °C.[40]

Malovana *et al*. compared liquid–liquid extraction with solid-phase extraction of phenolic compounds from wine samples.[41] The authors observed that sample preparation with liquid–liquid extraction using diethyl ether as extraction solvent at pH 2.0 provided optimum extraction yields of the phenolic compounds.[41] In other study, Valle Martinez-Ortega *et al*. compared the influence of different sample preparation treatments such as temperature, pH, addition of antioxidants, and use of anticoagulants on extraction of wine phenolic compounds from human plasma.[42] The results suggested that plasma samples should be stored below −20 °C before analysis and 1% ascorbic acid plus 10 μL mL^{-1} of *o*-phosphoric acid should be added to the sample matrix prior to extraction.[42] The recovery values for most phenolic compounds were better when enzymatic activity in plasma was inhibited, and acidified ethanol was used for deproteinization.

The eggplant, birch leaf, and wine phenolic examples, along with several other studies reported by different researchers,[43–45] justify a critical need for optimization and documentation of sample selection, preservation, and preparation methodologies that are often considered 'as a means to an end' by many researchers.

In other publications from our laboratory,[46,47] we have outlined a systematic approach for extraction of phytochemicals. The initial step is to check for the existence of multiple forms of the analyte of interest. It may not always be possible to extract multiple forms of an analyte of interest with a single extraction solvent or solvent mixture. One may require multiple solvent mixtures to extract different forms of varying polarities of conjugated mixtures. The second step is to select an extraction technique that will enable the researcher to efficiently extract the analyte of interest from the sample matrix. The third step will be evaluation of extraction solvents and/or solvent mixtures, as polarity matching between the analyte of interest and extraction solvent is critical for optimum extraction. The fourth logical step is optimization of extraction conditions (extraction temperature, number of extraction cycles, matrix particle size and

J Sci Food Agric **86**:2266–2272 (2006)
DOI: 10.1002/jsfa

solid-to-solvent ratio, flush volume, pressure, and static time) which are directly dependent on the availability of the extraction technology. Optimization of extraction parameters not only increases extraction efficiency of the analyte of interest but also reduces the solvent consumed and the waste generated during an extraction process.

Several studies from other researchers on optimization of extraction of polyphenols from apples,[48,49] anthocyanins and total phenolics from dried red grape skin,[50] isoflavones from soybeans,[51] tannin from infant foods,[52] and other bioactive phytochemicals from different matrices have also been published.[53–56] One can apply statistical experimental designs to minimize the number of experiments and simultaneously evaluate the influence of multiple factors to optimize a sample preparation procedure.[49]

Unfortunately, many peer-reviewed articles either frequently overlook or fail to document the details of the initial three analytical steps (sampling, sample preservation, and sample preparation). In addition, it seems many journal editors and referees do not demand that authors substantiate their efforts to control these critical steps for publication of reports on food composition. Four recent reviews on this subject further substantiate the need for optimization of the sample preparation procedures for accurate quantitation of phenolic compounds in different matrices.[22,30–32] Accurate analysis of phenolic constituents is essential for precisely quantifying the amount of phenolic compounds present in different foods, establishing appropriate dietary intake guidelines, and understanding their role as it relates to health and nutrition. Hence one should provide details of all three analytical steps in the experimental section of the manuscript dealing with food or phytochemical analysis. Detailed documentation of the initial three analytical steps is essential for reproducing results presented in the published literature.

CONCLUSIONS

The above examples with phenolic compounds demonstrate that optimization of sampling, sample preservation and sample preparation parameters are critical for accurate estimation of potentially bioactive phytochemicals present in different functional foods or food products. Testing in our laboratory and others demonstrates the influence of various sample preparation steps that need to be optimized for precise analysis of any phytochemicals extracted from different plant or food based matrices. Accurate estimation of bioactive compounds will enable researchers to correctly evaluate the role of such phytochemicals as they relate to health and provide precise dietary and safety guidelines on consumption of these phytochemicals. In addition, accurate quantitation of the bioactive components in functional foods will allow manufacturers, consumers, and marketing professionals to differentiate quality value-added products from the conventional ones. Most importantly, the scientific community, including journal editors and referees, must recognize the necessity of validating sampling, preservation, and extraction as critical steps in describing the content of bioactives in foods, in order to advance the field of functional foods for both research and industry.

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